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Competitive Enzymatic Interactions Determine the Relative Amounts of Prostaglandins E_2 and D_2

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Running title: Competitive formation of PGE_2 and PGD_2 from PGH_2

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Abbreviation: PG, prostaglandin; PGI_2, prostaglandin I_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; mPGES-1, microsomal PGE synthase-1; PGH_2, prostaglandin H_2; PGE_2, prostaglandin E_2; LC-MS/MS, liquid chromatography-tandem mass spectrometry; BMDM, bone marrow-derived macrophage; PGD_2, prostaglandin D_2; H-PGDS, hematopoietic PGD synthase; L-PGDS, lipocalin PGD synthase; COX, cyclooxygenase; AA, arachidonic acid; PGG_2, prostaglandin G_2; TXA_2, thromboxane A_2; 6-keto-$PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; TXB_2, thromboxane B_2; mPGES-2, microsomal PGE synthase-2; cPGES, cytosolic PGE synthase; NSAIDs, non-steroidal anti-inflammatory drugs; LPS, lipopolysaccharide; BHT, butylated hydroxytoluene; Hanks' Balanced Salt solution, HBSS; fetal bovine serum, FBS; Dulbecco's modified Eagle's medium, DMEM; SRM, selected reaction monitoring; TOF, time-of-flight; L929 cell-conditioned medium, LCC medium; ROS, reactive oxygen species; LGE_2, levuglandin E_2; LGD_2, levuglandin D_2; GST, glutathione-S-transferase.

INFLAMMATION, IMMUNOPHARMACOLOGY, AND ASTHMA

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Abstract

Prostaglandins (PG) are a family of cellular messengers exerting diverse homeostatic and pathophysiologic effects. Recently, several studies reported significant increases of PGI_2 and $PGF_{2\alpha}$ following inhibition of microsomal PGE synthase-1 (mPGES-1) expression, which indicated that PGH_2 metabolism might be re-distributed when the PGE_2 pathway is blocked. To address the determinants that govern the relative amounts of PGs, we developed an *in vitro* cell-free method, based on liquid chromatography-tandem mass spectrometry (LC-MS/MS), to measure the exact amounts of these PGs formed in response to the addition of recombinant isomerases and their selective inhibitors. Our *in vitro* cell-free assay results were confirmed in cells using bone marrow-derived macrophage (BMDM). Initially, we determined the *in vitro* stability of PGH_2 and noted that there was spontaneous non-enzymatic conversion to PGD_2 and PGE_2. Microsomal PGE synthase-1 markedly increased the conversion to PGE_2 and decreased conversion to PGD_2. Reciprocally, the addition of hematopoietic (H-PGDS) or lipocalin (L-PGDS) PGD synthase resulted in a relative increase of PGD_2 and decrease of PGE_2. A detailed titration study showed that the ratio of PGE_2/PGD_2 was closely correlated with the ratio of PGE synthase/PGD synthase. Our re-distribution results also provide the foundation for understanding how PGH_2 metabolism is re-distributed by the presence of distal isomerases or by blocking the major metabolic outlet, which could determine the relative benefits and risks resulting from interdiction in non-rate limiting components of PG synthesis pathways.

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Introduction

Cyclooxygenase (COX) enzymes, also known as PGH_2 synthases, catalyze the oxygenation of arachidonic acid (AA) to PGG_2, followed by the reduction of PGG_2 to PGH_2 which serves as a common substrate for various distal isomerases that generate five distinct primary PGs, PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TXA_2), of which 6-keto-$PGF_{1\alpha}$ and TXB_2 are the main stable non-enzymatic products of PGI_2 and TXA_2, respectively (Figure 1). These PGs consist of a series of extracellular and intracellular messengers that produce diverse physiologic effects on pain (Zeilhofer, 2007), inflammation and fever (McAdam et al., 2000), allergy (Prttipher, 2007), platelets (FitzGerald, 1991), cardiovascular system (Vane, 1983), cancer growth (Wang et al., 2007), renal function (Hebert et al., 2005), reproduction (Weems et al., 2006), and possibly Alzheimer's disease (McGeer and McGeer, 1999). In many cases, different PGs have counter-regulatory effects. For example, in contrast to PGE_2, PGD_2 in the brain has a role in promoting sleep (Smyth et al., 2009). Furthermore, various PGs have the potential to both promote and counteract inflammatory processes in the body, especially in acute allergic inflammation. Thus, the exact physiologic or pathophysiologic response is dependent on the relative amounts of biologically active PG species.

After the enzymatic conversion of PGH_2 was reported (Christ-Hazelhof et al., 1976), each PG specific isomerase was discovered and purified, including PGE synthase, PGD synthase, PGF_α synthase, PGI synthase, and TX synthase. Humans express three isoforms of PGE synthase, which are mPGES-1, mPGES-2 and cytosolic PGE synthase (cPGES). Whereas mPGES-2 and cPGES are constitutively expressed *in vivo*, mPGES-1 is of particular interest because it has been shown to be the most potent (Tanikawa et al., 2002) among PGE synthases and is induced by various stimuli including inflammatory signals in various cells and tissues

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(Guay et al., 2004). CAY10526 (4-(benzo[b]thiophen-2-yl)-3-bromo-5-hydroxy-dihydro-furan-2(3H)-one, $C_{12}H_7BrO_3S$) and CAY10589 (2-[[4-[([1,1'-biphenyl]-4-ylmethyl)amino]-6-chloro-2-pyrimidinyl]thio]-octanoic acid, $C_{25}H_{28}ClN_3O_2S$) are synthetic compounds that have been reported to be selective inhibitors of mPGES-1 (Guerrero et al., 2007; Koeberle et al., 2008). PGD synthase activity is also comprised of two isozymes, H-PGDS and L-PGDS. HQL-79 (4-(diphenylmethoxy)-1-[3-(1H-tetrazol-5-yl)propyl-piperidine, $C_{22}H_{27}N_5O$) is a selective H-PGDS inhibitor (Matsushita et al., 1998), and AT-56 (4-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-1-[4-(2H-tetrazol-5-yl)butyl]-piperidine, $C_{25}H_{27}N_5$) is reported to be a selective L-PGDS inhibitor (Irikura et al., 2009).

Non-steroidal anti-inflammatory drugs (NSAIDs) competitively interfere with binding of the AA substrate to COX enzymes. Treatment with traditional NSAIDs such as aspirin and ibuprofen decrease PGE_2 biosynthesis by non-selectively inhibiting both COX-1 and COX-2 (Garavito and Mulichak, 2003). However, serious gastrointestinal toxicity (Smyth et al., 2009) occurs at ordinary clinical doses due to their non-selective inhibition of both COX enzymes. There are also unintended consequences of selective inhibition of COX pathway enzymes. For example, rofecoxib and celecoxib are relatively selective for COX-2 and were developed under the assumption that specific inhibition of COX-2 would be anti-inflammatory and analgesic but lack gastrointestinal toxicity (Garavito and Mulichak, 2003). However, these COX-2 selective inhibitors are associated with an increased risk of hypertension, cardiovascular disease and stroke (Grosser et al., 2006; Timmers et al., 2007). This is, at least in part, because inhibition of COX-2 also blocks the production of PGI_2 and alters the balance between the vasoconstrictive properties of TXA_2 and vasodilatory properties of PGI_2 (Crofford et al., 2000; Bombardier et al., 2000). The recognition of these unintended consequences of individually blocking COX-1 and

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COX-2 has redirected efforts to examine the effect of interdiction in the individual downstream isomerases of PGH_2, especially on the inhibition of mPGES-1 (Guerrero et al., 2007; Mbalaviele et al., 2010; Rörsch et al., 2010). Since PGH_2 serves as a common substrate for at least five distinctive PGs and PGH_2 itself is extremely unstable and short-lived, it would be premature to consider mPGES-1 as a promising and safe new therapeutic target without further investigation and understanding of the effect of inhibition of mPGES-1 on the entire PG cascade. In order to address this important issue, we developed an *in vitro* cell-free assay system using LC-MS/MS to measure the formation of PGE_2 and PGD_2, to determine the global consequence of the addition of recombinant isomerases and the impact of selective inhibition on the relative production of PGE_2 and PGD_2 , which was also examined in cells using BMDM. We hypothesized that there would be a reciprocal relationship between the production of PGE_2 and PGD_2 when inhibiting their specific isomerases that could be biologically important.

Methods

Animals. For bone marrow experiments, C57BL/6 mice (6 to 12-wk-old, 25-35 g) were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed in a temperature-controlled room with a 12:12-h light-dark cycle and given standard chow and tap water. All studies involving mice were approved by the Institutional Animal Care and Use Committee and complied with the Animal Welfare Act.

Materials. Ovine COX-1, human recombinant COX-2, human recombinant mPGES-1, human recombinant H-PGDS, human recombinant L-PGDS, GSH, CAY10526, CAY10589, HQL-79, AT-56, AA, PGH_2, PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TXB_2, PGI_2 (sodium salt), 15-

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keto-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGE_2, 8-iso PGE_2, d_4-PGE_2 and d_4-PGD_2 were purchased from Cayman Chemicals (Ann Arbor, MI). Hemin, epinephrine, Tris base, hydrogen peroxide, citric acid, EDTA, and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). Monosodium phosphate, disodium phosphate, hydrochloric acid, and butylated hydroxytoluene (BHT) were purchased from Thermo Fisher Scientific (Rockford, IL). Formic acid was purchased from EMD Chemicals (San Diego, CA). Purified water was prepared using a Millipore (Billerica, MA) Milli-Q purification system or an ELGA (Saint Maurice Cedex, France) PURELAB Ultra purification system. Hanks' Balanced Salt solution (HBSS), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Mediatech (Manassas, VA). All organic solvents were HPLC grade or better and were purchased from Thermo Fischer (Rockford, IL), and all other chemicals and solvents were ACS reagent grade, unless stated otherwise.

The *in vitro* system. All experiments were carried out using an *in vitro* system based on a COX functional assay (Cao et al., 2010; Cao et al., 2011). Briefly, 20 μL of 25 mM GSH for mPGES-1 or 20 μL of 10 mM GSH for H-PGDS and L-PGDS was mixed on ice with 138 μL of 100 mM Tris•HCl buffer (pH 8.0, 37°C). Next, 20 μL of Tris•HCl buffer containing mPGES-1, H-PGDS or L-PGDS was added and incubated on ice for 2 min. A 2 μL aliquot of enzyme inhibitor in DMSO was added, and the solution was pre-incubated at 37°C in an Eppendorf Thermomixer R for 10 min. Each reaction was initiated by adding 20 μL of 20 μM PGH_2 (2 μM final concentration) in Tris•HCl buffer as substrate and terminated after 30 min by adding 50 μL of 2 M HCl. In place of PGH_2, 20 μL aliquots of 20 μM PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, or TXB_2 (final concentration 2 μM) were added to separate tubes as quantitative controls which

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contained the same solution composition but without enzymes. The quantitative controls represented the maximum amounts of each prostaglandin that could be formed if PGH_2 were quantitatively converted to a single product. After measurement using LC-MS/MS, the ratios of the peak areas of each prostaglandin in the experiments to the corresponding quantitative control were determined and expressed as percentages of the maximum theoretical yield.

d_4-PGE_2 and d_4-PGD_2 (10 μL; 100 ng/mL each in methanol/water, 50:50, v/v) were added as internal standards to correct for sample losses, degradation or changes in mass spectrometer response. Each sample was extracted using 800 μL hexane/ethyl acetate (50:50, v/v), and the organic phase was removed, evaporated to dryness under nitrogen gas, and reconstituted in 100 μL methanol/water (50:50, v/v) immediately prior to quantitative analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The peak areas were measured by using Applied Biosystems Analyst software (Foster City, CA) and manually inspected. The curves and data fitting were plotted by using Graphpad Prism 5 (Mountain View, CA), and other calculations were carried out using Excel (Microsoft Office) or Numbers (Apple iWork).

Mass spectrometry. For the quantitative analysis of all prostaglandins except for PGH_2, HPLC separations were carried out using a Shimadzu (Columbia, MD) Prominence HPLC system with a Waters (Milford, MA) XTerra MS C_{18} (2.1 mm × 50 mm, 3.5 μm) analytical column and a 5-min isocratic mobile phase consisting of acetonitrile/aqueous 0.1% formic acid (37:63, v/v) at a flow rate of 200 μL/min. As shown in Figure 2, all five derivatives of PGH_2 were resolved to baseline in less than 4 min using these chromatographic conditions. The HPLC system was interfaced to an Applied Biosystems API 4000 triple quadrupole mass spectrometer which was operated using negative ion electrospray. PGH_2 was measured using LC-MS/MS with

an 11-min linear gradient from 33% to 90% acetonitrile in aqueous 0.1% formic acid. Using gradient LC-MS/MS, PGH_2 eluted at a retention time of 7.7 min.

The PGs formed abundant $[M–H]^-$ carboxylate ions during negative ion electrospray, which were fragmented using collision-induced dissociation with nitrogen as a collision gas. The collision energy (-24 to -30 V) was optimized for each PG to maximize the formation of product ions for detection using selected reaction monitoring (SRM). Isomeric PGE_2, PGD_2 (Cao et al., 2008) and PGH_2 were measured using a SRM transition of *m/z* 351 to *m/z* 271 and the SRM transition of *m/z* 353 to *m/z* 193 was selected for $PGF_{2\alpha}$ (Dahl and van Breemen, 2010). The SRM transition of *m/z* 369 to *m/z* 163 was used for 6-keto-$PGF_{1\alpha}$, and the transition of *m/z* 369 to *m/z* 169 was used for the measurement of TXB_2. Similarly, the SRM of the transition of *m/z* 355 to *m/z* 275 was selected for the internal standards d_4-PGE_2 and d_4-PGD_2 (Cao et al., 2008).

High resolution negative ion electrospray tandem mass spectra of PGH_2 and its metabolites were acquired using a Waters Synapt G1 quadrupole time-of-flight (QqTOF) hybrid tandem mass spectrometer with a Waters Alliance 2690 HPLC system or a Shimadzu ion trap-TOF mass spectrometer with a Prominence HPLC system. HPLC separations were carried out as described above except that the mobile phase consisted of an 11-min linear gradient from 33% to 90% acetonitrile in aqueous 0.1% formic acid.

Cell culture assay. Although the *in vitro* assay provided information regarding biological mechanisms of action, the results might not necessarily reflect *in vivo* processes or even the situation within a cell. Therefore, the BMDM was utilized in which mPGES-1 and H-PGDS (L-PGDS) could be selectively inhibited in order to observe the re-distribution of PGH_2 metabolism.

BMDM was isolated from the rear legs of sacrificed C57BL/6 mice. The harvested rear legs were soaked in HBSS containing 2% heat-inactivated FBS under aseptic conditions. The

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bone marrow cells were obtained by flushing the tibias and femurs using HBSS and cultured in DMEM supplemented with 10% FBS, 10% L929 cell-conditioned (LCC) medium, 100 U/mL penicillin and 100 μg/mL streptomycin. After 72 hrs of cultivation, the non-adherent cells were removed by changing the medium. Adherent cells were subsequently propagated in culture. Cells were split at day 7 by EDTA (1 mM) and plated at a density of 5 × 105/mL into 6-well plate with LCC medium. On day 8, the cell culture medium was changed to DMEM medium containing 1% FBS, penicillin and streptomycin for 1.5 h prior to the addition of inhibitors CAY10526, CAY10589, HQL-79 and AT-56 adding 2 hrs prior to the LPS (1 μg/mL) treatment. Cells were incubated with 5% CO_2 humidified air at 37°C. The cell supernatants were collected and stored at -80°C until analysis after 16 hrs treatment with LPS and with different inhibitors. LPS was used to stimulate BMDM to activate COX-2 production and prostaglandin synthesis (Xiao et al., 2008).

Each supernatant was spiked with d_4-PGE_2 and d_4-PGD_2 as internal standards, and citric acid and BHT were then added to prevent free radical-catalyzed peroxidation. PGs were extracted using hexane/ethyl acetate. After centrifugation, the upper organic phase was collected and evaporated to dryness. Immediately before analysis using LC-MS/MS, each extract was reconstituted in methanol/water (Cao et al., 2008). Standards for calibration curves and quality control measurements were prepared by spiking cell culture medium with measured amounts of PGE_2 and PGD_2. These standards were then processed as described above. The concentrations of PGE_2 and PGD_2 in these standards ranged from 0.1 to 1000 ng/mL (Cao et al., 2008).

Statistical analysis. Samples were run in triplicate, and values are expressed as mean ± SD. Statistical significance was assessed using one-way analysis of variance (ANOVA), and *p*-values <0.05 were considered to indicate significant differences for all statistical tests.

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Results

Validation of enzyme and PGH_2 purity. To ensure the purity of each enzyme, ovine COX-1, human COX-2, mPGES-1, H-PGDS, and L-PGDS were incubated with AA and PGH_2 at 37°C for 10 min, respectively, and the production of PGs was then determined. Ovine COX-1 and human COX-2 did not increase the formation of PGE_2 or PGD_2 from PGH_2 compared with the control (Figure 3A), suggesting that neither ovine COX-1 or human COX-2 was contaminated with PG isomerases. In addition, the isomerases mPGES-1, H-PGD and L-PGDS had no effect on the metabolism of AA in the absence of ovine COX-1 or human COX-2 (Figure 3B), suggesting that these isomerases were not contaminated with either ovine COX-1 or human COX-2. The 2 μM PGH_2 solution used in these experiments was also analyzed using LC-MS/MS and was found to already contain 6.1 ± 1.1 % PGE_2, 1.8 ± 0.7 % PGD_2 and 0.2 ± 0.1 % $PGF_{2\alpha}$ due to non-enzymatic rearrangement.

***In vitro* system optimization.** PBS buffer (pH 6.0 to 8.0, 37°C) and Tris•HCl (pH 7.0 to 8.0, 37°C) produced similar results during incubations with PGH_2, but Tris•HCl buffer (pH 8.0, 37°C) produced slightly larger enzymatic yields and is recommended by Cayman Chemicals. Therefore, Tris•HCl buffer (pH 8.0, 37°C) was used for all subsequent experiments. GSH has been reported to function as an essential co-factor for three isomerases (mPGES-1, H-PGDS, L-PGDS) (Ouellet et al., 2002; Hohwy et al., 2008; Herlong and Scott, 2005). In our hands, GSH enhanced PGs formation only slightly for mPGES-1 and L-PGDS, and it was not essential for the function of these enzymes. For H-PGDS, however, GSH was indispensable (Table 1). GSH concentrations (0.1 to 12.5 mM) were varied among three isomerases, but did not affect reaction

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rates significantly. Thus, according to recommendations of the supplier Cayman Chemicals, 2.5 mM GSH was used for mPGES-1, and 1 mM GSH was used for H-PGDS and L-PGDS. The optimal levels of mPGES-1, H-PGDS and L-PGDS were approximately 3 units/μL, 0.1 unit/μL and 1 unit/μL, which was at the upper part of the linear range of the dose response curve. A pre-incubation time of 10 min was used prior to adding the substrate to allow the inhibitors to interact fully with the enzyme and to reach maximal inhibition potency (Cao et al., 2010; Cao et al., 2011). A reaction time of 30 min was used for enzymatic conversion of PGH_2 to PGs. A substrate dose-response curve was generated that indicated the production of each PG product after 30 min reached a plateau at approximately 4 μM of substrate. A substrate concentration of 2 μM corresponded to 50% response and was determined to be the K_m of the substrate.

Identification of PGH_2 and characterization of unknown derivatives. During the LC-MS/MS analysis of a PGH_2 standard (monitoring the SRM transition of *m/z* 351 to *m/z* 271), five peaks were observed as shown in Figure 4. The first two peaks were identified according to comparison with standards as PGE_2 and PGD_2, and the last peak was confirmed to be PGH_2 since it continued decreasing while other peaks increased over 160 min (Figure 5). The two remaining peaks (retention times 5.7 min and 6.3 min) remain unidentified and are not (by comparison with standards) PGI_2, 15-keto $PGF_{2\alpha}$, 13,14-dihydro-15-keto PGE_2, or 8-iso PGE_2. In a separate experiment, the half-life of PGH_2 in Tris•HCl buffer at pH 8 and 37°C was determined to be 5 min (Figure 6).

For additional characterization of the unknown peaks, LC-MS/MS analysis was carried out using high resolution QqTOF and IT-TOF mass spectrometers. All five compounds separated as shown in the chromatogram in Figure 4 were isomeric with identical elemental compositions of $C_{20}H_{32}O_5$. During tandem mass spectrometry on these mass spectrometers using different

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collision energies, all five compounds produced similar tandem mass spectra without any characteristic fragmentation that could distinguish them.

***In vitro* non-enzymatic conversions of PGH_2.** Since PGH_2 is extremely unstable and has a short half-life in aqueous solution, we used LC-MS/MS with SRM to determine the main degradation products of PGH_2 and their relative yields. In the absence of any enzyme, PGH_2 spontaneously converted to PGE_2, PGD_2, and a small amount of $PGF_{2\alpha}$ but no 6-keto-$PGF_{1\alpha}$ or TXB_2. Therefore, neither PGI_2 nor TXA_2 was spontaneously produced from PGH_2. The yields of PGE_2, PGD_2 and $PGF_{2\alpha}$ were approximately 44%, 15% and 1.6%, respectively, with a constant ratio among these species of ~3:1:0.1. We observed an unexpected phenomenon after optimizing the reaction conditions. There was nearly a 100% increase in PGD_2 formation from PGH_2 after the addition of 1 mM or 2.5 mM GSH, and for $PGF_{2\alpha}$, there was a 600% increase. However, PGE_2 formation was slightly decreased or not affected. Moreover, by adding an equivalent amount of H_2O_2, the effect of GSH addition on PGD_2 and $PGF_{2\alpha}$ was totally eliminated (Figure 7A) indicating that reactive oxygen species (ROS) might have a regulatory role in governing the relative amounts of these prostaglandins.

***In vitro* enzymatic conversions of PGH_2.** Since PGE_2 and PGD_2 play significant physiological roles in humans and are predominant products of PGH_2, we examined the roles in their formation of three commercially available human recombinant distal enzymes, mPGES-1, H-PGDS and L-PGDS. When the highest concentration of mPGES-1 (300 unit/μL) was incubated with PGH_2, the yield of PGE_2 increased to 62%, whereas PGD_2 decreased to 3.6% and $PGF_{2\alpha}$ decreased to basal level (Figure 7A). Similarly, when either the highest concentration of H-PGDS (120 unit/μL) or L-PGDS (10 unit/μL) was incubated with 2 μM PGH_2, the yield of

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PGD_2 increased to 78%, while the PGE_2 formation decreased to 10.8% and $PGF_{2\alpha}$ decreased to basal level (Figure 7B).

We next examined the impact of the addition of pharmacologic inhibitors of these downstream enzymes by employing the commercially available mPGES-1 selective inhibitors CAY10526 and CAY10589, H-PGDS selective inhibitor HQL-79, and L-PGDS selective inhibitor AT-56 in our *in vitro* system. After incubating PGH_2 with mPGES-1 (3 units/μL) and 300 μM CAY10526 or 100μM CAY10589, PGE_2 formation decreased to the basal level 45%, whereas PGD_2 formation increased to 25%, and $PGF_{2\alpha}$ increased only slightly (Figure 7A). Incubation with the H-PGDS specific inhibitor HQL-79 (50 μM) inhibited approximately 90% of PGD_2 formation (decreased to 26%) in the presence of H-PGDS (0.1 unit/μL), whereas PGE_2 formation increased to 38%, and $PGF_{2\alpha}$ decreased to basal level (Figure 7B). Similarly, the L-PGDS specific inhibitor AT-56 (100 μM) completely prevented PGD_2 formation (decreased to 24%) when incubating PGH_2 with L-PGDS (1 unit/μL), whereas PGE_2 formation increased to 40% and $PGF_{2\alpha}$ decreased to basal level (Figure 7B). The effects of these inhibitors on other isomerases were also tested. Surprisingly, CAY10526 and CAY10589 were less specific than expected and also inhibited both H-PGDS and L-PGDS *in vitro*. In contrast, HQL-79 had no inhibitory effect on either L-PGDS or mPGES-1, and AT-56 inhibited neither H-PGDS nor mPGES-1 (Table 2).

***In vitro* re-distribution assay.** As obvious shifts of PGH_2 to other PGs were observed using inhibitors to block their corresponding isomerases, we predicted that there would be re-distribution in products by inhibition of isomerases that compete for the substrate PGH_2. We first mixed different volumes of mPGES-1 (3 units/μL) and H-PGDS (0.1 unit/μL) or (L-PGDS, 1 unit/μL) in the same system. As a result, with more mPGES-1 activity, more PGE_2 and less

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PGD_2 were produced, and vice versa (Figure 8) in a predictable fashion according to the ratio of PGES to PGDS activity. We also examined the impact of pharmacologic inhibition with HQL-79 and AT-56 to further determine the consequence of re-distribution by inhibiting H-PGDS and L-PGDS, respectively (Figure 9). As expected, by increasing the concentrations of HQL-79 or AT-56, conversions of PGH_2 moved toward the production of PGE_2, whereas the production of PGD_2 decreased. Since our data showed that the purported mPGES-1 inhibitors CAY10526 and CAY10589 also strongly inhibited H-PGDS and L-PGDS, re-distribution tests by inhibiting mPGES-1 with these agents did not produce re-distribution of PGH_2 metabolism *in vitro*.

Cell culture re-distribution assay. CAY10526 and CAY10589 did not produce re-distribution of PGH_2 metabolism in BMDM since these agents inhibited not only mPGES-1 but also H-PGDS and L-PGDS. AT-56 had no effect on decreasing PGD_2 production, which was not surprising since L-PGDS protein expression was not detectable in BMDM (Xiao unpublished data). Therefore, only H-PGDS was available for inhibition by pharmacologic intervention in BMDM. Similar to the *in vitro* re-distribution assay, treatment of BMDM with the H-PGDS inhibitor HQL-79 altered PGH_2 conversion toward the production of PGE_2, while the production of PGD_2 decreased (Figure 10).

Discussion

Previously, we reported that a COX functional assay (Cao et al., 2011) used to screen COX-2 selective inhibitors results in production of both PGE_2 and PGD_2, which raised the obvious question as to how PGE_2 and PGD_2 are produced in the absence of downstream isomerases. We initially suspected that the COX-2 enzyme was contaminated with certain

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isomerases or that it might have some unknown functions which could catalyze conversion of PGH_2 to PGs. When incubating COX-1 or COX-2 with PGH_2 produced no increase in the production of the PGs, we then redirected our focus on investigating the stability of the intermediate PGH_2. These studies showed that PGH_2 was extremely unstable in aqueous solution *in vitro* and spontaneously and non-enzymaticly converted to PGE_2, PGD_2 and $PGF_{2\alpha}$, with a constant ratio of ~3:1:0.1 (Figure 7). This not only provided an explanation for the results in our COX functional assay but also clarified the identities and yields of PGH_2 products. Based on our *in vitro* findings, it is possible that PGH_2 would also convert to PGs *in vivo* without any downstream isomerases, which further brings into question the efficacy of inhibiting PGE_2 formation using mPGES-1 inhibitors.

The pivotal finding of our study is the PGH_2 metabolic re-distribution to other forms of PGs by inhibiting either PGE synthase or PGD synthase (Figure 7). A possible explanation is that mPGES-1 and H-PGDS compete for the substrate PGH_2 so that inhibition of one enzyme would shunt PGH_2 to the other. In order to prove this explanation, several different approaches were used. Titration studies (Figure 8) showed how the conversion of PGH_2 was closely correlated with the relative levels of PGE synthase and PGD synthase. A similar re-distribution phenomenon (from PGD_2 to PGE_2) was also observed *in vitro* (Figure 9) when using the H-PGDS selective inhibitor HQL-79 or the L-PGDS selective inhibitor AT-56, since the major outlet PGD_2 was blocked. Moreover, the BMDM cell culture study (Figure 10) confirmed the PGH_2 metabolism re-distribution in cells by inhibiting H-PGDS. Purported to be mPGES-1 selective inhibitors, CAY10526 and CAY10589 also inhibited PGD synthase and could not be used to study re-distribution toward PGD_2. PGH_2 metabolism re-distribution also explains the

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reported increases of PGI_2 and $PGF_{2\alpha}$ by inhibition of mPGES-1 in mice (Guay et al., 2004; Mbalaviele et al., 2010; Rörsch et al., 2010; Trebino et al., 2005).

In the near future, our observations could probably be used to provide therapeutic guidance for diseases involving PGs, because the re-distribution and accumulation of certain types of PGs might be beneficial and useful for treatment. For example, higher levels of PGI_2 and $PGF_{2\alpha}$ would be favorable since they have anti-inflammatory effects, and more PGD_2 could have anti-inflammatory effects (Smyth et al., 2009). Since various PGs can produce opposing effects, imbalance among them might cause unexpected problems or risks that must not be ignored such as with the use of highly selective COX-2 inhibitors. For instance, an increase of PGI_2 might cause side effects such as low blood pressure in certain patients and even result in certain chronic and fatal diseases which are not easy to observe (Smyth et al., 2009).

We observed moderate inhibition of PGE_2 formation by CAY10526 *in vitro*, however, CAY10526 was reported to be a strong mPGES-1 selective inhibitor (Guerrero et al., 2007). The probable reason (Guerrero et al., 2007) is that CAY10526, which caused a significant reduction in PGE_2 production in the induction phase, did not affect PGE_2 formation in the post-induction phase, which means that CAY10526 selectively inhibited mPGES-1 protein expression.

During the identification of PGH_2 (Figure 4), two peaks (retention times 5.7 min and 6.3 min) remained unidentified but were not (by comparison with standards) PGI_2, 15-keto $PGF_{2\alpha}$, 13, 14-dihydro-15-keto PGE_2, or 8-iso PGE_2. Some possible structures include the reactive γ-keto aldehydes, levuglandins E_2 (LGE_2) and D_2 (LGD_2) (Boutaud et al., 1999). All of these compounds are isomers of PGH_2.

GSH has been reported to be an essential co-factor for mPGES-1 (Ouellet et al., 2002), H-PGDS (Hohwy et al., 2008), and L-PGDS (Herlong and Scott, 2005). Our studies confirmed

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the indispensability of GSH for H-PGDS, but it was not necessary for either mPGES-1 or L-PGDS in our hands (Table 1). This indicated that GSH was not an essential co-factor for mPGES-1 and L-PGDS in our *in vitro* cell-free assay if these two isomerases were not contaminated with GSH. During the non-enzymatic conversion study of PGH_2, a 600% increase of $PGF_{2\alpha}$ production and an almost 100% increase of PGD_2 production were found when adding GSH into PGH_2 standard in aqueous solution (Figure 7A). It has been reported that GST increases $PGF_{2\alpha}$ formation, which mainly results from the GST (glutathione-S-trandferase) enzymatic catalysis (Burgess et al., 1987). However, other reducing agents, like GSH, can also increase $PGF_{2\alpha}$ production (Christ-Hazelhof et al., 1976; Burgess et al., 1999; Nugteren and Christ-Hazelhof, 1980), which indicates that this is a non-enzymatic process, possibly by serving as a specific electron donor promoting the conversion of PGH_2 to $PGF_{2\alpha}$ (Keeting et al., 1987). However, little is known about the increase of PGD_2 by adding GSH, which raised our interest in the undiscovered differences between PGE_2 and PGD_2. Since both PGE_2 and PGD_2 have the same elemental composition and similar structure, it is very curious that they behave so differently in the presence of GSH or other reagents, and these issues are being addressed by our on-going studies.

In summary, although mPGES-1 inhibitors could be used as alternatives to COX-2 inhibitors to specifically decrease the amount of PGE_2, they could also cause PGH_2 metabolism re-distribution. Thus, it is necessary to have a full understanding of the mechanisms of action and physiological effects of all PGs. The consequences of selective inhibition of one or more prostaglandin isomerases determine possible subsequent risks. Recently, mPGES-1 was found to be functionally coupled with COX-2 (Murakami et al., 2000), which means that mPGES-1 would normally catalyze the transformation of PGH_2 produced by COX-2. Then, using one or more

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inhibitors to partially inhibit COX-2 and strongly inhibit mPGES-1 might be a more reasonable therapeutic approach. Alternatives might include PGE_2 receptor antagonists or high affinity PGH_2 analogs.

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Authorship Contributions

Participated in research design: Yu, Xiao, Zhao, Christman, and van Breemen.

Conducted experiments: Yu, and Zhao.

Contributed new reagents or analytic tools: Xiao, Christman, and van Breemen.

Performed data analysis: Yu.

Wrote or contributed to the writing of the manuscript: Yu, Xiao, Zhao, Christman, and van Breemen.

Provided funding: Xiao, Christman, and van Breemen.

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Footnote

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Figure Legends

Figure 1. Scheme for the metabolism of arachidonic acid (AA) to form different prostaglandins (PGs).

Figure 2. Negative ion electrospray LC-MS/MS SRM chromatograms showing the detection of 1 μM PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TXB_2 standards in methanol/water (50:50, v/v). SRM transitions: 6-keto-$PGF_{1\alpha}$ *m/z* 369→163 (retention time 1.6 min); TXB_2 *m/z* 369→169 (retention time 2.2 min); $PGF_{2\alpha}$ *m/z* 353→193 (retention time 2.7 min); PGE_2, PGD_2 *m/z* 351→271 (retention times 3.2 and 3.7 min, respectively).

Figure 3. Ovine COX-1, human COX-2, mPGES-1, H-PGDS, and L-PGDS were incubated at 37°C for 10 min with substrate PGH_2 (A) or AA (B), respectively. PG formation (%) represents the sum of all PGs that were detected relative to the theoretical maximum based on the substrate PGH_2 or AA. All the values are the mean ± SD, n=3. *: $p < 0.05$, significance differences in PGs formation compared with no enzyme (A and B).

Figure 4. Negative ion electrospray SRM chromatograms of the transition *m/z* 351 to *m/z* 271 showing the detection of 2 μM PGH_2 standard in methanol/water (50:50, v/v) at pH 7 and 4°C, and its non-enzymatic derivatives using an 11-min linear gradient from 33% to 90% acetonitrile in aqueous 0.1% formic acid at a flow rate 200 μL/min. PGE_2 and PGD_2 (retention times 4.5 min and 4.8 min) were identified by comparison with standards. PGH_2 (retention time 7.7 min) was identified by monitoring its level over 160 min at pH 7 and 4°C. The remaining two peaks (retention times 5.7 min and 6.3 min) remain unidentified but were not (by comparison with standards) PGI_2, 15-keto $PGF_{2\alpha}$ or 13, 14-dihydro-15-keto PGE_2.

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Figure 5. Levels of PGE_2 (A), PGD_2 (B), PGH_2 (E) and two unknown compounds (C and D) formed over 160 min from an initial concentration of 2 μM PGH_2 in methanol/water (50:50, v/v) at pH 7 and 4°C. The formation of PGE_2 and PGD_2 was constant during the first 50 min and reached a plateau by 70 min (A and B), while the amount of PGH_2 continued to decrease over 160 min (E). The curves representing the unknown compounds had inflection points suggesting that these are unstable compounds.

Figure 6. PGH_2 decomposition curve in Tris•HCl buffer at pH 8 and 37°C (time points 1, 2, 3, 5, 7, 10, 15, 25, 40 min). Based on this curve, the half-life of PGH_2 was determined to be 5 min.

Figure 7. PGs formation from PGH_2 under different conditions. The initial concentration of PGH_2 was 2 μM; and the GSH and H_2O_2 concentrations were 1 mM for H-PGDS and L-PGDS or 2.5 mM for mPGES-1. mPGES-1, H-PGDS, and L-PGDS (units/μL); CAY10526, CAY10589, HQL-79, and AT-56 (μM). All values are mean ± SD, n=3.

Figure 8. The effects on PGE_2 and PGD_2 formation from 2 μM PGH_2 of different ratios of mPGES-1 and H-PGDS (A), or mPGES-1 and L-PGDS (B). PGE_2 formation increased when the proportion of mPGES-1 was increased; and PGD_2 formation increased when the proportion of H-PGDS or L-PGDS was increased. All values are expressed as the mean ± SD, n=3.
*: $p < 0.05$, significant differences in PGE_2 formation compared with mPGES-1 (0.3 units/μL)/H-PGDS (0.01 units/μL) (A) and mPGES-1 (0.3 units/μL)/L-PGDS (0.1 units/μL) (B).
#: $p < 0.05$, significant differences in PGD_2 formation compared with mPGES-1 (0.3 units/μL)/H-PGDS (0.01 units/μL) (A) and mPGES-1 (0.3 units/μL)/L-PGDS (0.1 units/μL) (B).

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Figure 9. The consequences of adding different concentrations of the H-PGDS selective inhibitor HQL-79 (A) or L-PGDS selective inhibitor AT-56 (B) to a system containing equivalent volumes of mPGES-1 (3 units/μL) and H-PGDS (0.1 unit/μL) or L-PGDS (1 unit/μL) incubated with 2 μM substrate PGH_2. All the values are expressed as mean ± SD, n=3. *: $p < 0.05$, significant differences in PGE_2 formation compared without addition of HQL-79 (A) or without addition of AT-56 (B). PGE_2 formation increased while PGD_2 formation decreased with increasing concentrations of HQL-79 or AT-56.

#: $p < 0.05$, significant differences in PGD_2 formation compared without addition of HQL-79 (A) or without addition of AT-56 (B).

Figure 10. The effect of the H-PGDS selective inhibitor HQL-79 on the LPS-stimulated production of PGE_2 and PGD_2 from BMDM (A). Values are the mean ± SD, n=3. *: $p < 0.05$, significant differences in PGE_2 formation compared with no HQL-79 addition; #: $p < 0.05$, significant differences in PGD_2 formation compared with no HQL-79 addition. PGE_2 formation increased and PGD_2 formation decreased with increasing concentrations of HQL-79.

Examples of LC-MS/MS analyses of PGE_2 and PGD_2 extracted from BMDM after treatment without or with 100 μM HQL-79 (B).

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Table 1. PGs formation from PGH_2 incubated with or without GSH and various enzymes. All values are mean ± SD, n=3. GSH at 2.5 mM was used for control and mPGES-1 incubations, while 1 mM GSH was used for incubations with H-PGDS and L-PGDS.

GSH addition	PGs formation, % (n=3)	
	PGE_2	PGD_2
Control w/o GSH	**44.3 ± 3.6**	**14.9 ± 1.5**
Control w/ GSH	**42.7 ± 2.9**	**24.2 ± 3.1**
mPGES-1 w/o GSH	**50.1 ± 3.3**	**14.7 ± 2.1**
mPGES-1 w/ GSH	**51.8 ± 3.4**	**15.1 ± 2.0**
H-PGDS w/o GSH	**42.4 ± 4.0**	**15.7 ± 3.2**
H-PGDS w/ GSH	**20.1 ± 2.1**	**48.0 ± 2.3**
L-PGDS w/o GSH	**19.7 ± 1.6**	**49.8 ± 3.1**
L-PGDS w/ GSH	**18.5 ± 2.0**	**50.8 ± 3.4**

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Table 2. The effects of mPGES-1 inhibitors CAY10526 and CAY10589, H-PGDS inhibitor HQL-79, and L-PGDS inhibitor AT-56 on conversion of PGH_2 to PGE_2 and PGD_2 by mPGES-1, H-PGDS and L-PGDS.

Inhibitor	PGs formation (% yield from initial 2 μM PGH_2)					
	mPGES-1 (3 units/μL)		H-PGDS (0.1 unit/μL)		L-PGDS (1 unit/μL)	
	PGE_2	PGD_2	PGE_2	PGD_2	PGE_2	PGD_2
No inhibitor	53.5 ± 3.7[a]	15.1 ± 0.9	20.3 ± 2.1	48.1 ± 3.0	18.2 ± 2.7	50.1 ± 2.8
CAY10526 (300 μM)	45.2 ± 2.5	24.9 ± 2.0	37.8 ± 1.8	26.7 ± 1.3	38.9 ± 2.6	28.1 ± 2.4
CAY10589 (100 μM)	44.9 ± 2.8	25.5 ± 1.6	40.8 ± 3.1	24.2 ± 1.8	40.4 ± 3.4	23.7 ± 0.8
HQL-79 (50 μM)	54.4 ± 2.1	14.6 ± 1.0	38.3 ± 2.0	26.4 ± 1.7	20.4 ± 1.5	49.1 ± 1.8
AT-56 (100 μM)	55.1 ± 3.4	14.7 ± 1.6	21.2 ± 1.4	47.3 ± 2.5	39.8 ± 2.2	24.1 ± 1.3

[a]All values are mean ± SD, n=3.

Figure 1

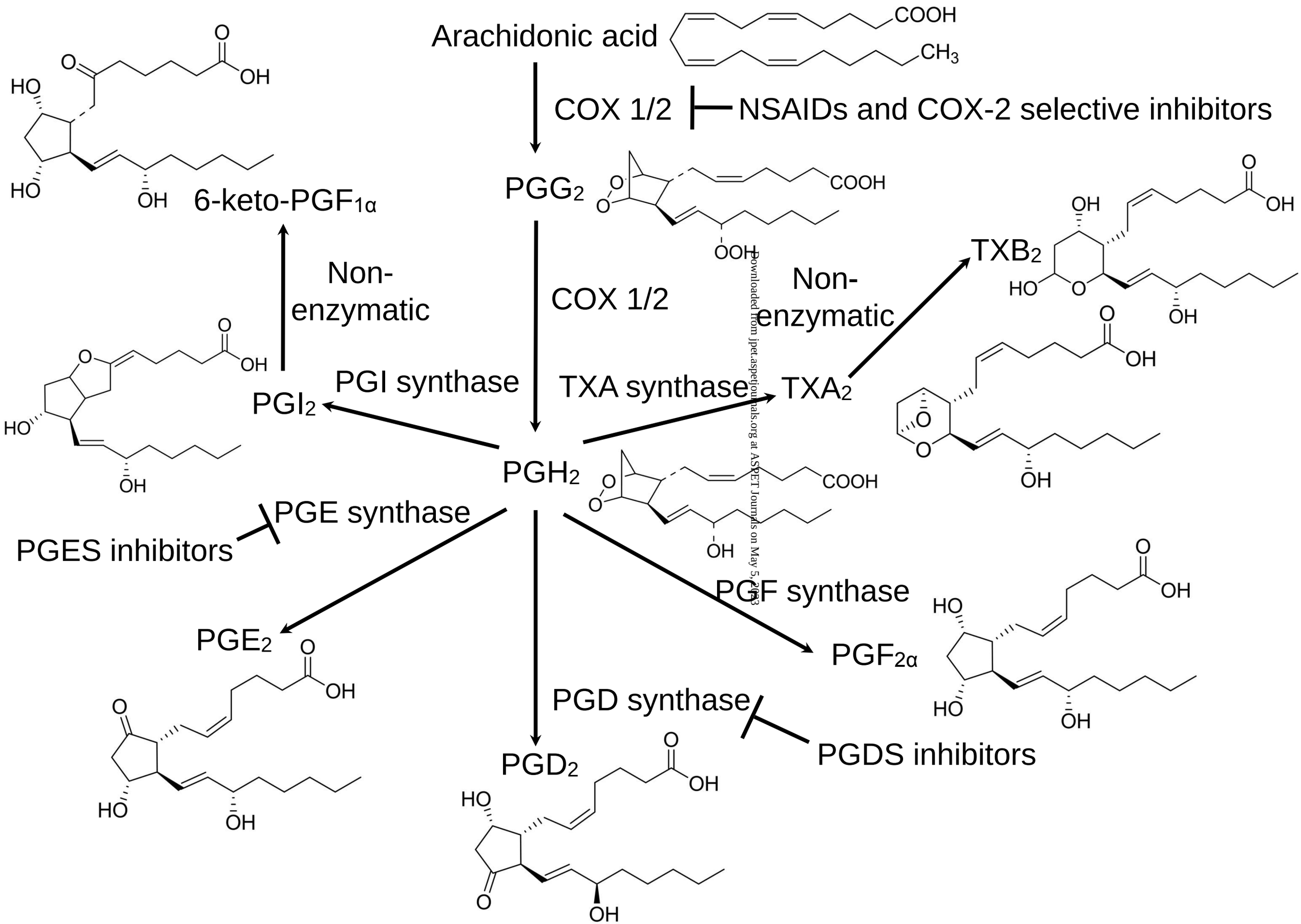

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Figure 2

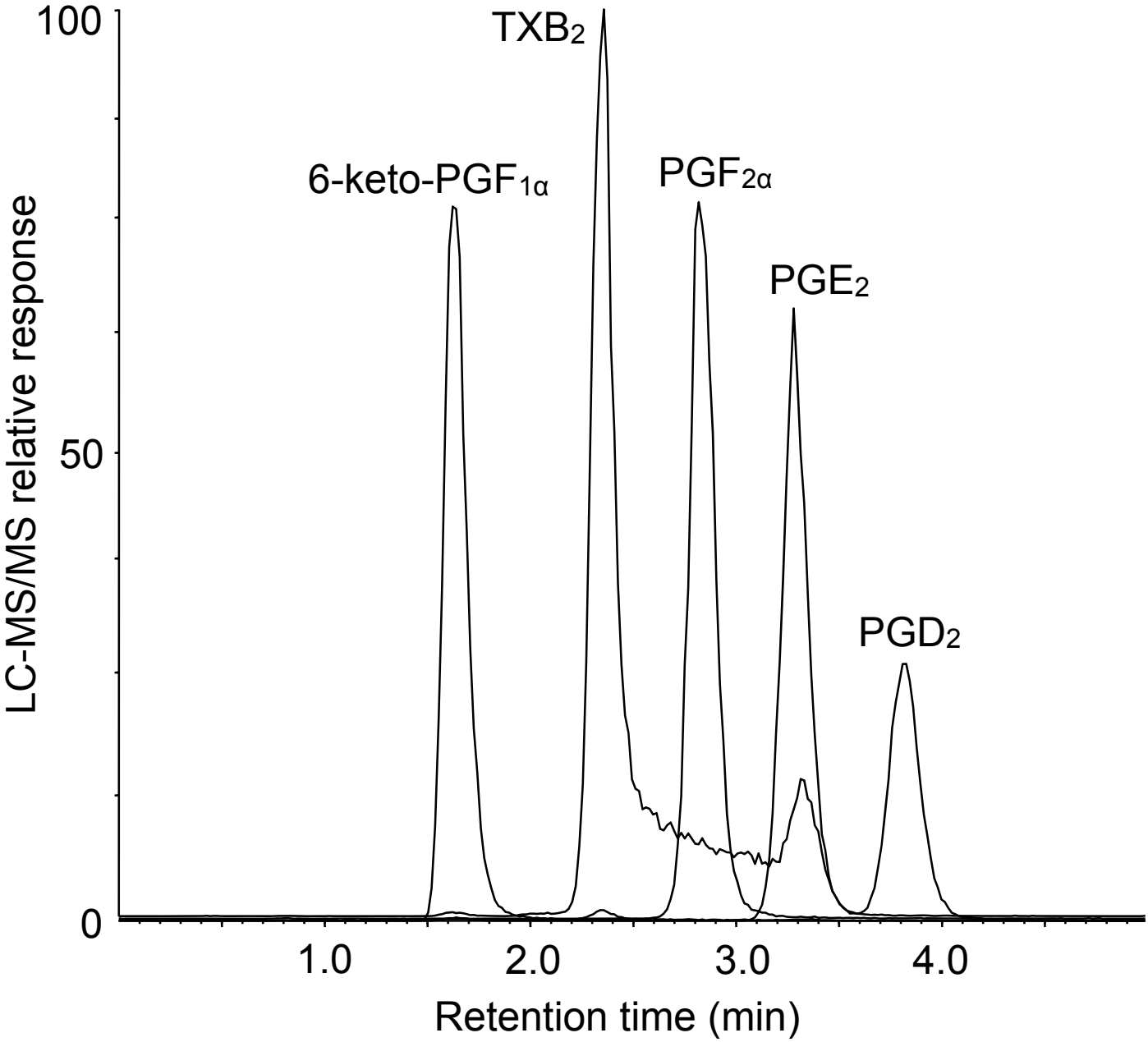

Figure 3

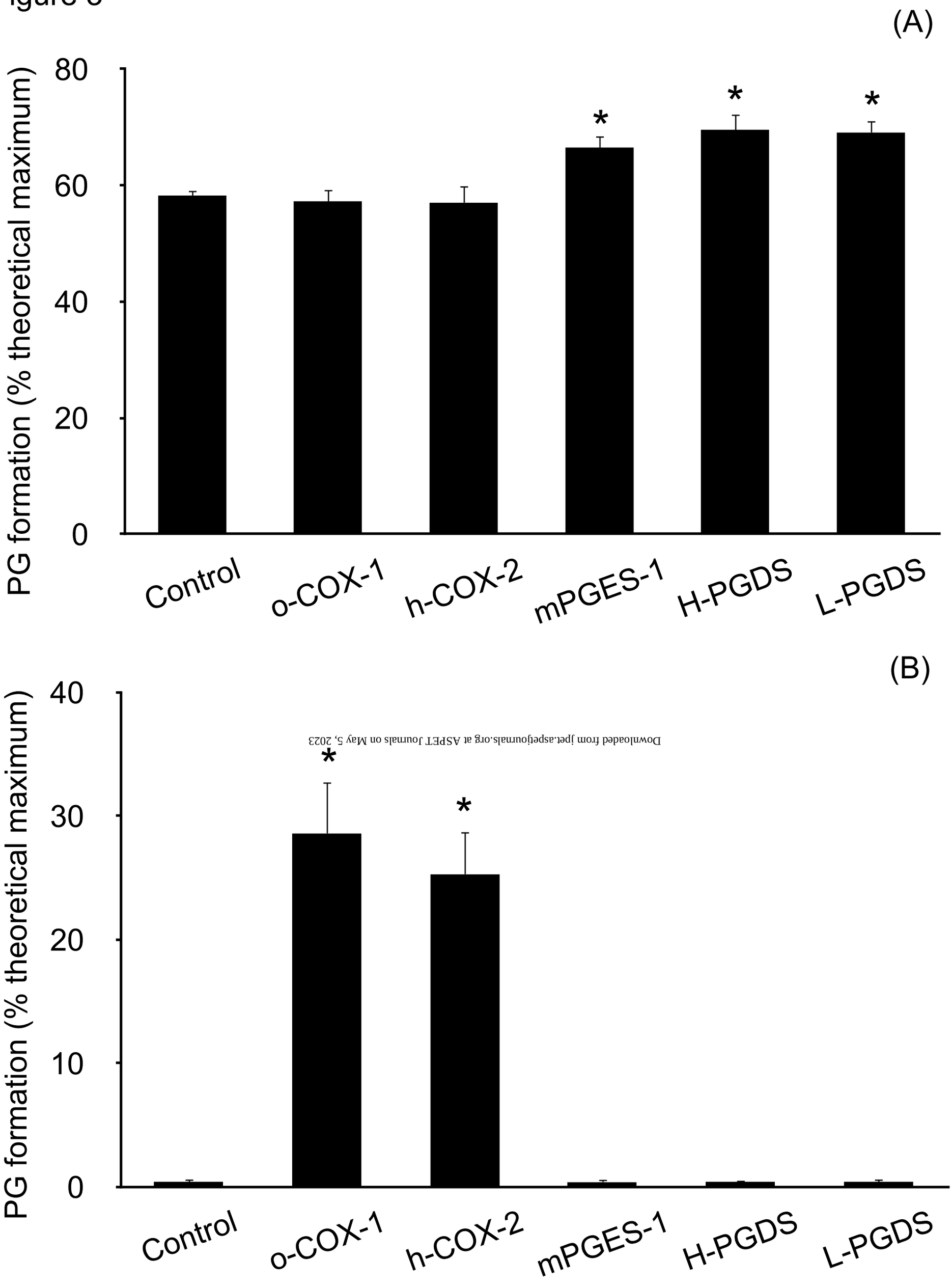

Figure 4

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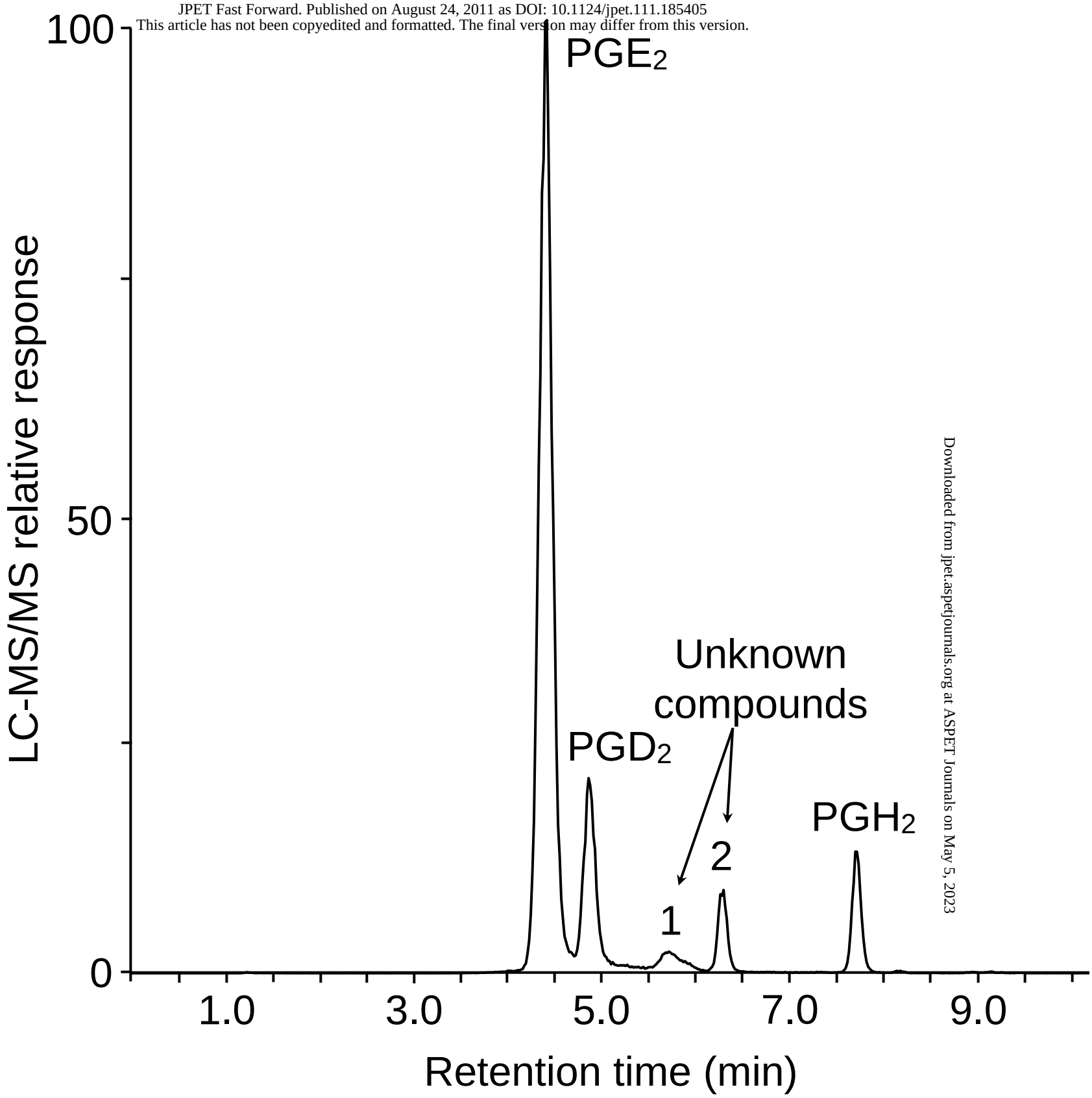

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Figure 5

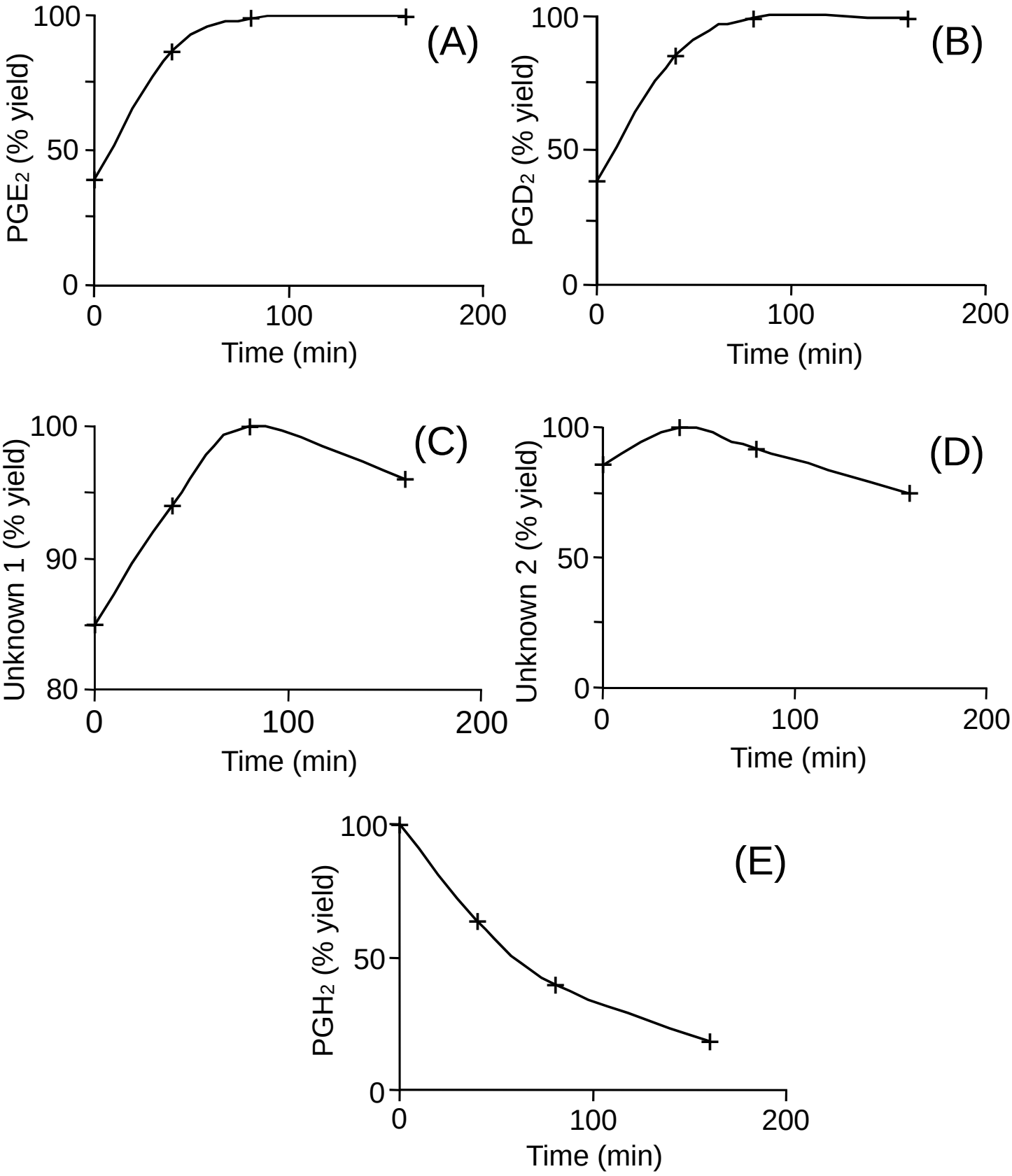

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Figure 6

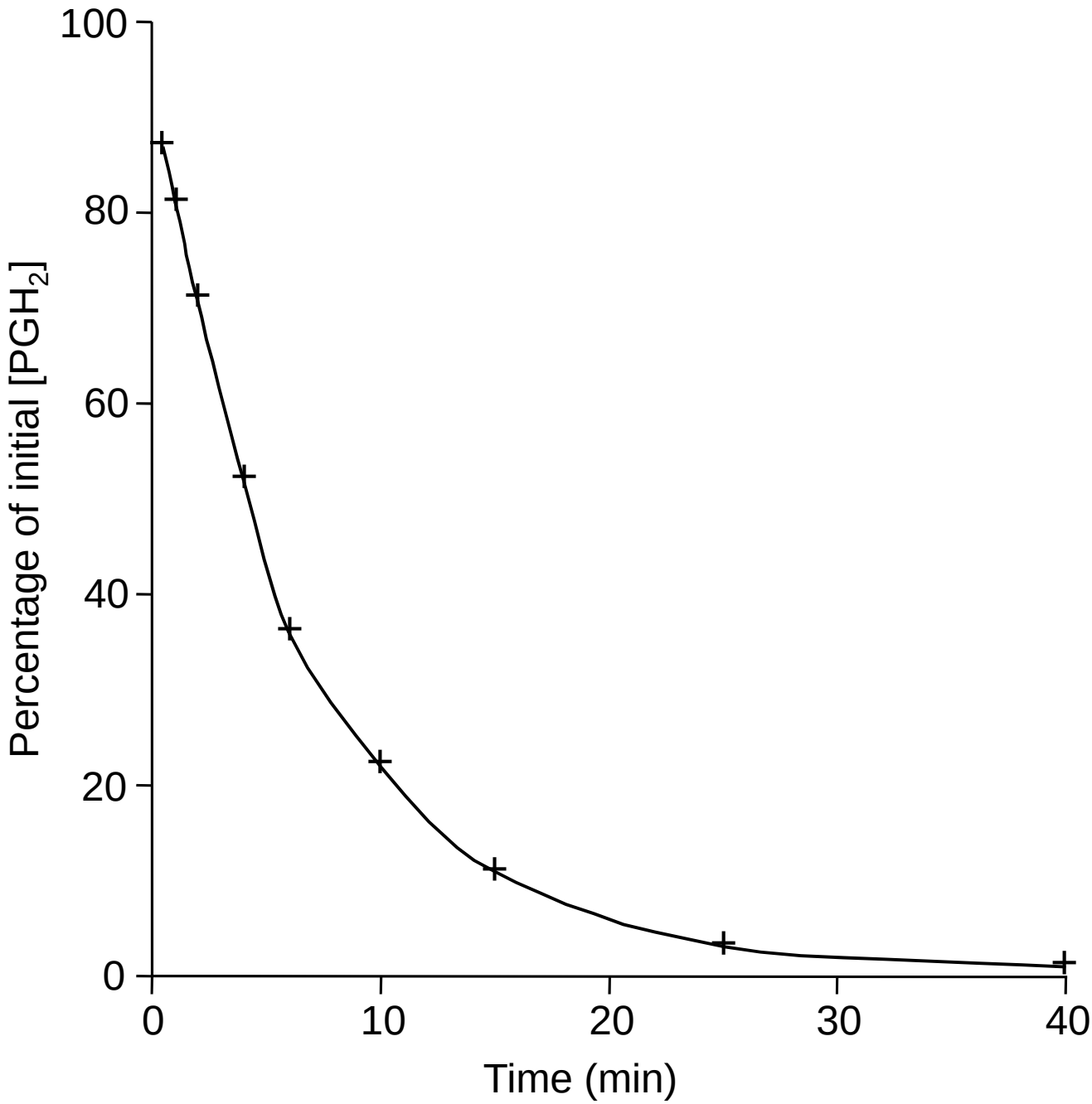

Figure 7

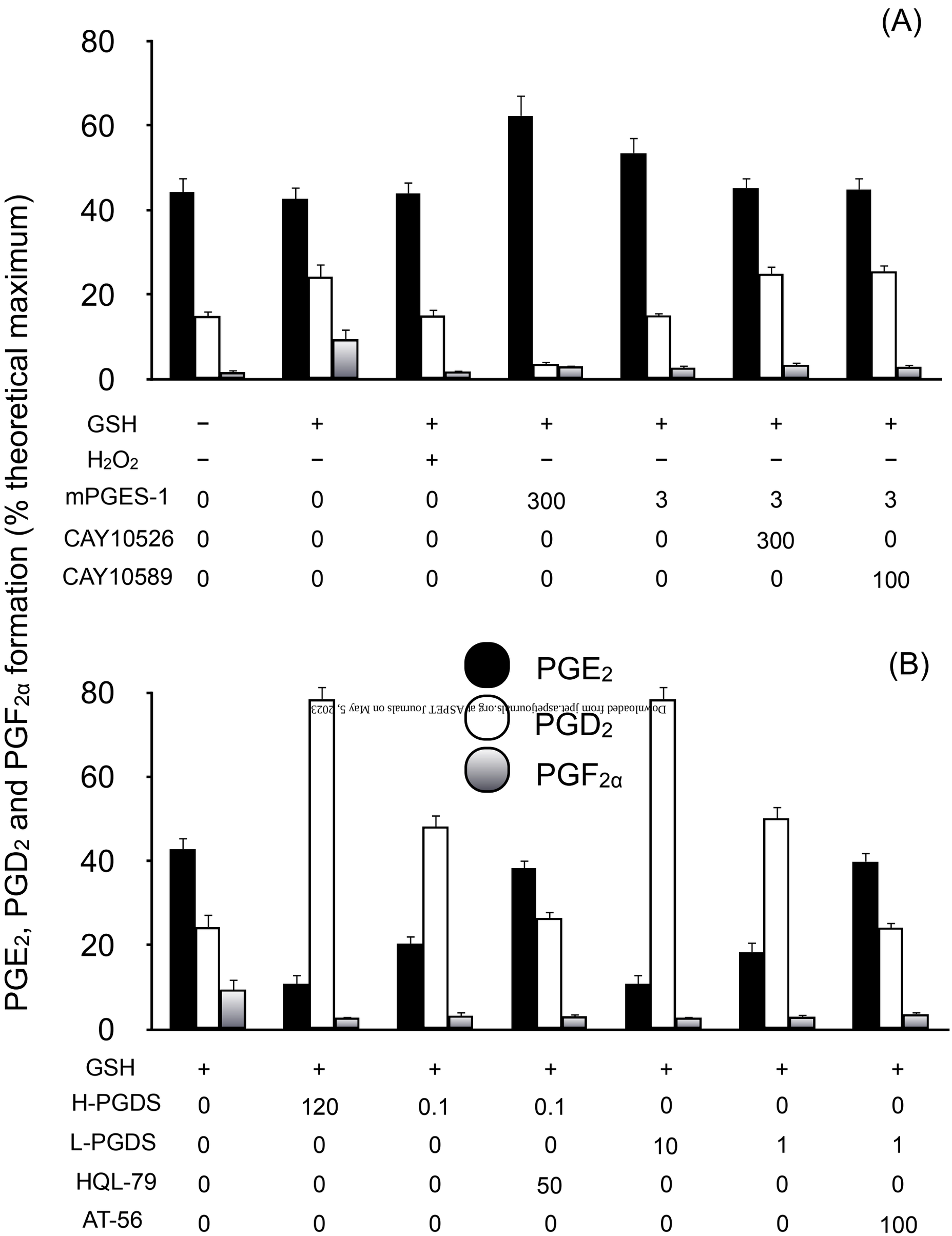

Figure 8

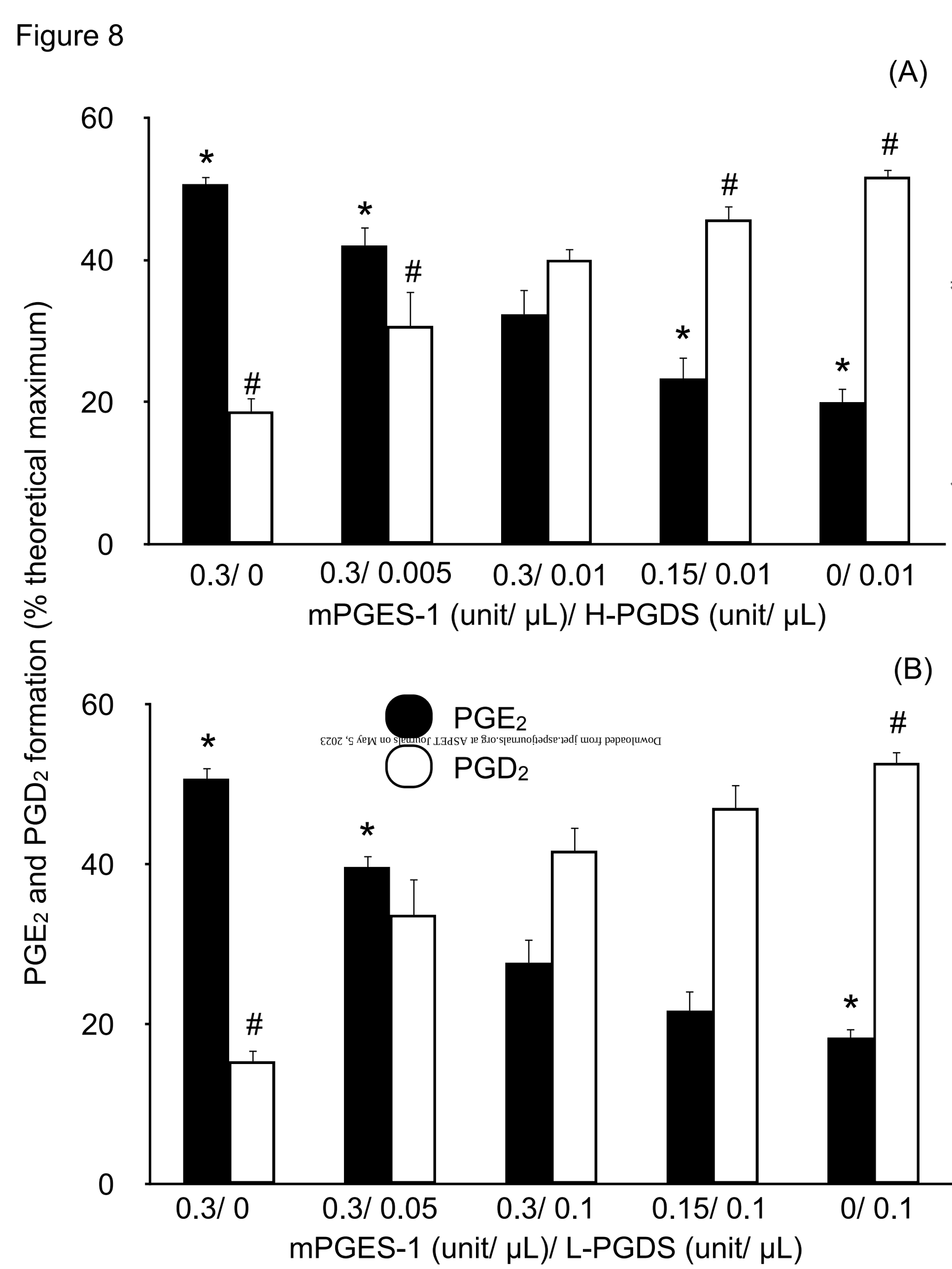

Figure 9

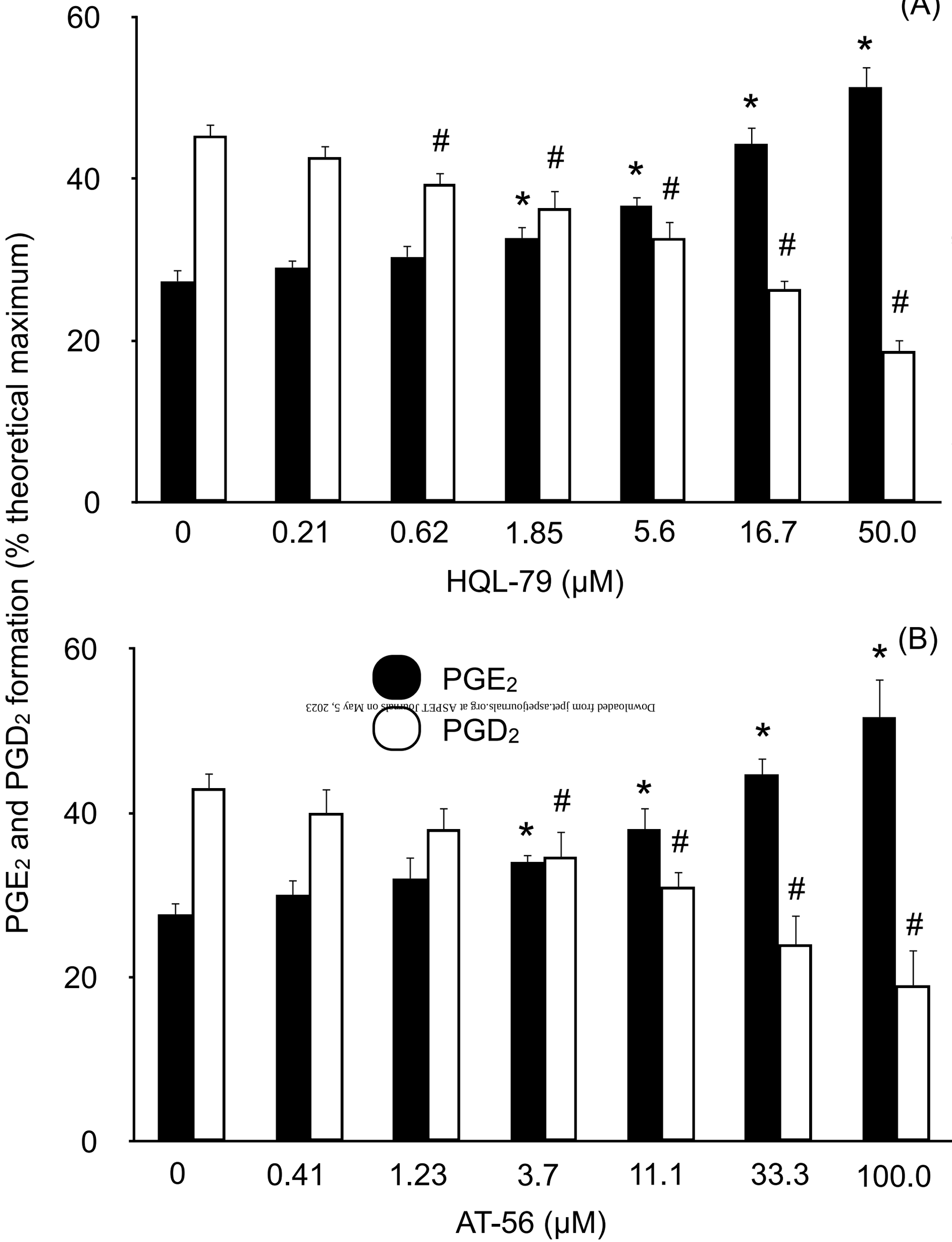

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Figure 10